EMBO reports

Proteasome mapping reveals sexual dimorphism in tissue-specific sensitivity to protein aggregations

Edmund Charles Jenkins, Nagma Shah, Maria Gomez, Gabriella Casalena, Dazhi Zhao, Timothy C Kenny, Sara Rose Guariglia, Giovanni Manfredi and Doris Germain

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 August 2019

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

I am sorry to say that the evaluation of your manuscript is not a positive one. As you will see, all three referees acknowledge that your analysis of sex- and tissue-specific differences in proteasome activity are more extensive than earlier reports and potentially interesting. Yet, they also raise a number of largely overlapping concerns in particular concerning the ER-alpha and disease link and the large variability of the data. Moreover, the referees note that the data are largely correlative and that the causality between proteasome activity and protein aggregation or ubiquitination has not been established.

Due to the nature of the criticisms, the amount of work likely required to address them, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from the referees upon initial assessment, I am afraid that we do not feel it would be productive to call for a revised version of your manuscript at this stage. We overall feel that the physiological relevance and the disease link would have to be substantially strengthened for potential publication in EMBO reports.

I am sorry to disappoint you on this occasion, and hope that the referee comments will be helpful in your continued work in this area.

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REFEREE REPORTS

Referee #1:

Tissue-specific differences in the level, subunit composition, and specific activity of the proteasome are known to exist, but in general they have been characterized only in passing. This study takes on this problem in earnest and the results prove to be interesting. The authors add substantially to our knowledge of the tissue-specific variations in proteasome activity, for example, showing that the intestine has activity levels far in excess of the many other tissues analyzed. Also interestingly, they document substantial sex differences in proteasome activity. Both observations have the potential to illuminate the significance of proteasome variations in human disease. Overall I think this is a very valuable paper, and, suitably revised, it may lead to further focus on these problems, which would be a welcome development on the field. Although my overall impression is clearly positive, I noticed some problems to be considered:

1. I think the authors do a good job of arguing for their main points. However, especially towards the end of the results section, they extend the argument too far. Consider Figs 5N and 5Q. Here the differences in proteasome levels are vanishingly small (~3% as I read the graphs). Whether or not such a small difference can be quantified accurately, it would in any case be of doubtful physiological significance. Needless to say, these graphs do not "go to zero" so the magnitudes of the differences appear larger than they are. I think the authors and the journal will be best served if this whole argument (related to doxa nd toER alpha) is eliminated from the paper.
2. The paper documents interesting sex differences in proteasome activity and ubiquitin-protein conjugate accumulation. This is seen in some tissues but not others. There is a feature of the data that I seem to resolve, which doesn't seem to be commented on in the text (sorry in advance if I missed it). That is, in some determinations, the parameter seems to vary more substantially in males than in females. A good example is Fig 2A (lung). Of the older males, three actually look comparable to the females, while three others are elevated more than 2-fold. I'd view the data of Fig 3B/C similarly. Note sure how universal or reproducible this is but it seems worthy of comment. It also prods me to think that some of the analyses done here would be more powerful if the same samples could be processed in parallel protocols, say to give you both proteasome activity and K48 chain levels.
3. The authors never run native gels to distinguish between 20S proteasomes and 26S proteasomes. This can be nontrivial, especially for tissue-derived samples, but it may be worth a try for the more interesting tissues such as intestine.
4. It would be good to be a bit more explicit about how the insoluble fractions are normalized.
5. I did not see a good definition of "total proteasome activity". Total proteasome activity over the organism is I'm skeptical that it's good to use this term. One could just refer to the activity level or specific activity on a tissue-by-tissue basis.
6. In spots the author argue that the proteasome "generally assumed to be a single entity." But I think that everyone who works on proteasomes is very well aware that this is not the case.....

Referee #2:

Zhao et al. observed that the proteasome activity across nine tissues, in male and female mice. From these results, the authors found that the chymotrypsin-like activity of the proteasome in the spinal cord, kidney, and intestine of male mice is lower than female mice. Next, the authors focused on sexual dimorphism in proteasome activity in spinal code and suggested that a sex-specific difference in proteasome activity is correlated with a higher susceptibility to accumulate protein aggregates under stress or pathological conditions. Finally, the authors tried to identify the pathway that enhances proteasome activity and rescue misfolded protein accumulation. They suggested that activation of the estrogen receptor alpha axis is critical to enhance proteasome activity and rescue misfolded protein accumulation.

The purpose of the study to clarify the mechanism for differences in protein quality control and lifespan between male and female is interesting. The authors nicely showed that male mice are prone to form protein aggregates. However, they fail to show evidence supporting the relationship between the difference in proteasome activity, the amount of protein aggregates, and ERα axis. The following are specific comments regarding these points.

1. Fig 1: The authors stated," the caspase-like activity could not be determined..." However, the

caspase-like activity was assayed in many previous papers. This reviewer wonders whether the samples the authors used were appropriate for observing a difference in proteasome activity. Also, the authors used Ac-GPLR-AMC as a substrate for caspase-like activity, according to Materials and Methods, but this reviewer does not think this substrate is appropriate for this purpose; instead it is for trypsin-like activity.
2. Fig 1A-D: This reviewer does not understand the significance of these figures. What does "total activity" mean? Because the relative contents of the proteasome, cell numbers, proliferation status are different between each tissue, the comparison of the proteasome activity like these figures does not make sense.
3. Fig 1E: Regarding the results in the male intestine, the data are so dispersed that this reviewer is not convinced of the soundness of the experiments.
4. Fig 1E-F: The sex-specific differences might arise from the difference in the ration of immunosubnits to standard subunits because immunoproteasomes exhibit higher chymotrypsin-like activity and lower caspase-like activity than standard core particles. The authors should quantify the amount of proteasome subunits, especially standard catalytic subunits (β1, 2, 5) and immunosubunits (β1i, 2i, and 5i) for each tissue by western blot.
5. Fig 1E-F: The activity of the proteasome in this kind of assay also depends on the integrity of the 26S proteasomes during sample preparation, the efficiency of extracting proteasomes from tissues, the association of activators like 11S Reg or PA28, and influence by other proteins. For example, hemin is a well-known proteasome inhibitor. Western blot for proteasome subunits in soluble fractions and pellet fractions, western blot for 11S, native-PAGE analysis of the lysates for separation of 26S and 20S followed by western blot and in-gel activity assay are needed. Also, assays in the presence of a low concentration of SDS (e.g., 0.02-0.03%) should be performed to understand whether the differences arise from 20S core particles.
6. Fig 1G-J: Because the ubiquitination activity and the mechanism for proteostasis should be different between each tissue, this reviewer does not agree with the authors' statement in line 7-10, page 6. The difference of the proteasome amount could be a cause, but the data never support that it is a significant cause. Also, for the same reason, this reviewer does not agree with the statement in line 12-17, page 6. The combination of peptidase assay and western for K48-ub is not an accurate reflection of the proteasome activity.
7. Fig 1G-J: the data are so dispersed that this reviewer is not convinced of the soundness of the experiments.
8. Fig 1K: This figure does not refer to differences between male and female and does not bring any implications in this context.
9. Suppl Fig 3: Western blot for tubulin in intestine samples is quite poor. The authors should load nearly the same amount of the samples in each lane.
10. Fig 2D: This reviewer cannot find data showing increased trypsin-like activity in aged mice than young mice. What is "a compensatory mechanism" in line 14, page 8? What mechanism are the authors assuming?
11. Fig 3B-J: The authors claim that differences in proteasome activities translate into differences in susceptibility to the accumulation of protein aggregates in line 13-15, page 9. The difference in the proteasome activity could be a cause, but the data never support that it is a significant cause.
12. Fig 3N-O: What proteins are shown in these figures? Is it probed with anti-SOD1-antibody? If not, the authors should observe aggregation of SOD1 and perform western blot for insoluble SOD1.
13. Fig 4B-G: The authors attribute the decrease in K48-ub to the increase in proteasome activity. However, it is hard to imagine that a mere few % increase in proteasome activity drastically reduces the accumulation of ubiquitinated proteins. There is no evidence showing that this increase in proteasome activity is involved in the clearance of ubiquitinated proteins. The y-axes of Figures 4D and 4G and 4Q should start at zero.
14. Fig 4N: The authors should explain why there was no difference between WT and ERα KO in female mice. The authors conclude that ERα promotes the activity of proteasome under mitochondrial proteotoxic stress, but there is no evidence. To conclude this, the authors should examine whether the increase in proteasome activity by Dox {plus minus} HS treatment is canceled in ERα KO mice. Also, the statement in the abstract "Importantly, activation of the estrogen receptor ...in critical to enhance proteasome activity and rescue misfolded protein accumulation in ALS spinal cords" is not at all supported by the data.
15. Suppl Fig 7: It is interesting that female mice are more capable of activating mitochondrial stress response genes. However, the data show poor response to Dox in contrast to a strong response to HS. The authors should explain this point. Also, statistical analysis should compare between male and female. In the text, the authors should spell out NRF1, OMI1, and ATF.

16. What is "LC" in Fig 3CG and 4CFPQ?
17. The title is an overstatement. The authors did not show susceptibility to protein aggregations in "other tissues."

Referee #3:

The manuscript by Jenkins and colleagues reports a sexual dimorphism in proteasome activity and in susceptibility to protein aggregation in CNS and intestine. They show that females have significantly higher activity in several tissues and that different tissues have different basal activity of the proteasome. Using mice subjected to heat shock and SOD1-G93A transgenic mice, they suggest that sex specific differences in proteasome activity translate into a higher susceptibility to accumulate protein aggregates in males under heat shock stress, while female were more protected, possibly explaining why female with ALS show a later onset of symptoms and survive longer than male. Finally, they authors show that doxycycline reduces the accumulation of K48Ub proteins and increases proteasome activity in both male and female spinal cord and activates the ERalpha axis of the UPRmt. They propose that the activation of the ERalpha axis by doxycycline is critical to enhance proteasome activity and rescue misfolded protein accumulation in ALS spinal cords.

The results of this manuscript reveal a sexual and tissue-specific difference in proteasome activity, which was already reported in the literature but only a small subset of tissues was tested. This study is more extensive which is of great interest to better understand how proteostasis is differentially regulated in different tissues and gender. The disease-related part is interesting but lacks statistical power to me, which associated to the experimental variability, sounds a bit less convincing. The importance of the ERalpha axis for the doxycycline regulation of proteasome activity also need to be strengthened. Proteostasis regulation in different genders and in pathophysiological conditions is of great interest to the field, but to me, the weaknesses of this study need to be addressed before being considered for publication.

Specific comments:

1. The authors have measured proteasome activity in vitro with or without the proteasome inhibitor bortezomib only in Suppl. Fig. 1, while all experiments should have been conducted +/- proteasome inhibitors to be more accurate. In the same line, one of the big problems for the identification of ubiquitinated proteins in cellular lysates is the high activity of deubiquitinases (DUBs). The authors have analysed the load of K48-linked polyubiquitin chains without DUBs inhibitors (50mM Tris, 250mM NaCl, 5mM EDTA, 0.5% NP-40, 50mM NaF, 1mM DTT plus protease inhibitors) which could be one possible explanation for the observed variability among mice groups.

2. The authors concluded that the differences in activity are not due to the amount of proteasome by monitoring the levels of beta5 subunit, but the total level of proteasome subunits doesn't always correlate with proteasome levels as these subunits might belong to the pool of unassembled subunits, proteasome intermediates, free 20S proteasomes or singly- and doubly-capped proteasomes. Native gels should be able to confirm their statement, as the different forms of proteasomes can be identified.

3. The authors state: "Collectively these results suggest that sex specific differences in proteasome activity translate into a higher susceptibility to accumulate protein aggregates in males under heat shock stress (Fig. 3J)"; however, no real evidence indicate that this statement in true, as protein aggregates are mainly in the insoluble fractions and no significant differences are observed between insoluble fractions from male and female intestines while this tissue harbours the biggest gender difference in proteasome activity. This should be discussed by the authors.

4. The authors state: "Taking advantage of samples obtained from spinal cords of ERalpha knockout mice28, we confirmed that in absence of ERalpha spinal cord proteasome activity is decreased (Fig. 5N). This observation therefore confirms that the ERalpha promotes the activity of the proteasome under mitochondrial proteotoxic stress". As the authors have not tested proteasome activity in WT and ERalpha KO mice under mitochondrial stress, thus this statement needs to be modified.

5. The authors see a drastic effect of doxycycline on the load of K48-linked ubiquitin chains (Fig.

4B) while the activity of proteasome increases only by about 4% in female (Fig. 4D). It is clear that a small modification of proteasome activity can have important effects on the clearance of proteins but an alternative scenario is that doxycycline by inhibiting mitochondrial translation and possibly cytosolic translation (Mortison et al, Cell Chemi. Biol. 2018), decreases the synthesis of unwanted proteins and therefore their subsequent K48-specific ubiquitinylation. This could be discussed in the discussion part.

Minor comments:

1. The authors state: "Collectively, this analysis revealed that the decline in proteasome activity during aging is not uniform among tissues or sexes and that the overall decline is less pronounced in females than in males as reduction in proteasome activity is observed in 4 tissues in females but in 6 tissues in males (Fig. 2E, F)". This is not appropriate to me, as there are 3 tissues where proteasome activity is increased in old female, while 6 are increased in older males. Thus, the opposite conclusion could also be made. Moreover, the Fig. 2F shows that trypsin-like proteasome activity is decreasing in old males compared to younger males, while based on Suppl. Fig. 4B, it is the opposite. This, need to be corrected.

2. The authors say that HS in mice mimics high fever and this needs to be supported by references.

3. It is not clear whether statistical analyses are missing in Suppl. Fig. 5B and 5D or whether the results are not significant.

1st Revision - authors' response 25 October 2019

Referee#1:

Tissue-specific differences in the level, subunit composition, and specific activity of the proteasome are known to exist, but in general they have been characterized only in passing. This study takes on this problem in earnest and the results prove to be interesting. The authors add substantially to our knowledge of the tissue-specific variations in proteasome activity, for example, showing that the intestine has activity levels far in excess of the many other tissues analyzed. Both observations have the potential to illuminate the significance of proteasome variations in human disease. Overall I think this is a very valuable paper, and, suitably revised, it may lead to further focus on these problems, which would be a welcome development on the field. Although my overall impression is clearly positive, I noticed some problems to be considered:

1. I think the authors do a good job of arguing for their main points. However, especially towards the end of the results section, they extend the argument too far. Consider Figs 5N and 5Q. Here the differences in proteasome levels are vanishingly small (~3% as I read the graphs). Whether or not such a small difference can be quantified accurately, it would in any case be of doubtful physiological significance. Needless to say, these graphs do not "go to zero" so the magnitudes of the differences appear larger than they are. I think the authors and the journal will be best served if this whole argument (related to doxa nd toER alpha) is eliminated from the paper.

Response: In agreement with the reviewer's suggestion, the data related to dox and ER alpha has been removed.

2. The paper documents interesting sex differences in proteasome activity and ubiquitin-protein conjugate accumulation. This is seen in some tissues but not others. There is a feature of the data that I seem to resolve, which doesn't seem to be commented on in the text (sorry in advance if I missed it). That is, in some determinations, the parameter seems to vary more substantially in males than in females. A good example is Fig 2A (lung). Of the older males, three actually look comparable to the females, while three others are elevated more than 2-fold. I'd view the data of Fig 3B/C similarly. Note sure how universal or reproducible this is but it seems worthy of comment. It also prods me to think that some of the analyses done here would be more powerful if the same samples could be processed in parallel protocols, say to give you both proteasome activity and K48 chain levels.

Response: The variability in the proteasome and Ub-K48 chain in some tissues was addressed in the original version, but was shown in a supplementary figure; we have now moved this data in the main figure to make this point more clearly. In addition, we have conducted additional analysis to address the variability. We

focused mainly on the variability in the intestine because this is where it is most striking (Fig. 1E). We now show in supplementary figure 1B a principal component analysis of global gene expression in human intestine, showing that, while gene expression clusters nicely in females, there is wide variability in males. This data, therefore, supports the variability we observed in male mice in this tissue. In addition, while conducting the native gels requested by the reviewer, we found clear differences in the migration of the proteasome between males. Collectively these analyses have further convinced us that the variability is telling us something fundamental about the proteasome in male intestine. Further, as pointed out by this reviewer, there is also variability in selected tissues during aging (more variability in aged relative to young). We added the discussion of these differences in the text, as they may reveal an additional level of complexity to the regulation of the proteasome during aging.
Lastly, the proteasome and Ub-K48 protocols are indeed performed in parallel. In conclusion, the variability is not merely a technical issue, because in most samples the activity of proteasome and Ub-K48 levels are similar among mice. The variability is an intrinsic aspect of proteasome biology that needs to be considered in the future.

3. The authors never run native gels to distinguish between 20S proteasomes and 26S proteasomes. This can be nontrivial, especially for tissue-derived samples, but it may be worth a try for the more interesting tissues such as intestine.

Response: Native gels have been performed in both the intestine and spinal cord, the two tissues most investigated in our study. The gels are shown in the new panels 2F and 2G and the quantification of assembled versus unassembled proteasome shown in panels 2H and 2I.

4. It would be good to be a bit more explicit about how the insoluble fractions are normalized.

Response: A new section describing the method of normalization of the insoluble fraction is now included in materials and methods.

5. I did not see a good definition of "total proteasome activity". Total proteasome activity over the organism is I'm skeptical that it's good to use this term. One could just refer to the activity level or specific activity on a tissue-by-tissue basis.

Response: This was meant as a different way to look at the data, but clearly not essential to the argument. Thus, panels 1A and B were removed.

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6. In spots the author argue that the proteasome "generally assumed to be a single entity." But I think that everyone who works on proteasomes is very well aware that this is not the case.....

Response: Agreed. The sentence was removed.

Referee #2:

The purpose of the study to clarify the mechanism for differences in protein quality control and lifespan between male and female is interesting. The authors nicely showed that male mice are prone to form protein aggregates. However, they fail to show evidence supporting the relationship between the difference in proteasome activity, the amount of protein aggregates, and ERα axis. The following are specific comments regarding these points.

1. Fig 1: The authors stated," the caspase-like activity could not be determined..." However, the caspase-like activity was assayed in many previous papers. This reviewer wonders whether the samples the authors used were appropriate for observing a difference in proteasome activity. Also, the authors used Ac-GPLR-AMC as a substrate for caspase-like activity, according to Materials and Methods, but this reviewer does not think this substrate is appropriate for this purpose; instead it is for trypsin-like activity.

Response: The peptide used is (Ac-GPLD-AMC, Enzo Life Sciences, cat No. BML-AW9560-0005) **sold by Enzo Life Sciences for studying caspase like activity. If it was for trypsin-like we would have detected the activity, but we did not. Perhaps others have seen caspase-like activity in fresh tissues. Nevertheless, we think that this does not change the conclusions of the manuscript.**

2. Fig 1A-D: This reviewer does not understand the significance of these figures. What does "total activity" mean? Because the relative contents of the proteasome, cell numbers, proliferation status are different between each tissue, the comparison of the proteasome activity like these figures does not make sense.

Response: This was meant as a different way to look at the data, but clearly not essential to the argument. Thus, panels 1A and B were removed.

3. Fig 1E: Regarding the results in the male intestine, the data are so dispersed that this reviewer is not convinced of the soundness of the experiments.

Response: The variability in male intestine was originally addressed in suppl. Fig. 2 but for clarity we have now brought this data in the main figure. The question of variability was also a concern of reviewer 1. To further address this concern we have conducted additional analysis to address the variability. We focused mainly on the variability in the intestine because this is where the variability was most striking (Fig. 1E). We now show in supplementary figure 1B a principal component analysis of gene expression in human intestine, which shows that, while gene expression clusters nicely in females, there is wide variability in males. This data, therefore supports the variability we observed in male mice. In addition, while conducting the native gels, we found clear differences in the migration of the proteasome among males. Collectively these analyses have further convinced us that the variability is telling us something fundamental about the proteasome in the intestine of males.

4. Fig 1E-F: The sex-specific differences might arise from the difference in the ration of immunosubnits to standard subunits because immunoproteasomes exhibit higher chymotrypsin-like activity and lower caspase-like activity than standard core particles. The authors should quantify the amount of proteasome subunits, especially standard catalytic subunits (β1, 2, 5) and immunosubunits (β1i, 2i, and 5i) for each tissue by western blot.

Response: Thus it is an intriguing issue, and we agree that immunoproteasome may contribute to these differences. However, the potential sources of variability that contribute to these observations are likely to be enormous and vary from one tissue to the other. Thus, we deem identifying them is beyond the scope of the current study, but will surely be a significant topic of analysis for years to come.

5. Fig 1E-F: The activity of the proteasome in this kind of assay also depends on the integrity of the 26S proteasomes during sample preparation, the efficiency of extracting proteasomes from tissues, the association of activators like 11S Reg or PA28, and influence by other proteins. For example, hemin is a well-known proteasome inhibitor. Western blot for proteasome subunits in soluble fractions and pellet fractions, western blot for 11S, native-PAGE analysis of the lysates for separation of 26S and 20S followed by western blot and in-gel activity assay are needed. Also, assays in the presence of a low concentration of SDS (e.g., 0.02-0.03%) should be performed to understand whether the differences arise from 20S core particles.

Response: The type of analysis would address the specific nature of each proteasome species in each tissue. While this is of interest and represents a future direction for the future, the goal of the current study is to identify differences in proteasomes that are tissues and gender specific.

6. Fig 1G-J: Because the ubiquitination activity and the mechanism for proteostasis should be different between each tissue, this reviewer does not agree with the authors' statement in line 7-10, page 6. The difference of the proteasome amount could be a cause, but the data never support that it is a significant cause. Also, for the same reason, this reviewer does not agree with the statement in line 12-17, page 6. The combination of peptidase assay and western for K48-ub is not an accurate reflection of the proteasome activity.

Response: We agree that these differences are very likely the combination of difference in proteasome activity and other aspect of protein quality control in tissues. This point is now more clearly stated in the discussion.
The sentence on page 6 was removed. We now simply state that there is a direct correlation between activity of proteasome and level of Ub-K48 in individual mice (new Fig. 2E)

7. Fig 1G-J: the data are so dispersed that this reviewer is not convinced of the soundness of the experiments.

Response: This point was addressed as stated above (point 3).

8. Fig 1K: This figure does not refer to differences between male and female and does not bring any implications in this context.

Response: This figure was the initial data to address the source of variability. We now have refined the study and present new data (principal component analysis in suppl. Fig.1B) for which information on sex is available and the native gels in figure 2F; so, the heatmap was moved to supplementary figure 4. The heatmap was generated from publicly available datasets for which information on sex is not available.

9. Suppl Fig 3: Western blot for tubulin in intestine samples is quite poor. The authors should load nearly the same amount of the samples in each lane.

Response: The western was redone, and actin used as reference protein.

10. Fig 2D: This reviewer cannot find data showing increased trypsin-like activity in aged mice than young mice. What is "a compensatory mechanism" in line 14, page 8? What mechanism are the authors assuming

Response: It is in suppl. Fig. 5. We agree with the reviewer, the mention of compensatory mechanism was removed, as we have no clear proof that this is the case.

11. Fig 3B-J: The authors claim that differences in proteasome activities translate into differences in susceptibility to the accumulation of protein aggregates in line 13-15, page 9. The difference in the proteasome activity could be a cause, but the data never support that it is a significant cause.

Response: Figures 1 and 2 establish that there are differences in proteasome activity between tissues and sexes; so, in figure 3, we are asking if these differences correspond to differences in protein aggregation under stress conditions.

12. Fig 3N-O: What proteins are shown in these figures? Is it probed with anti-SOD1-antibody? If not, the authors should observe
aggregation of SOD1 and perform western blot for insoluble SOD1.

Response: Sorry for the confusion. Yes, the blot was probed for SOD1, this has been clarified (figure 4N-O).

12. Fig 4B-G: The authors attribute the decrease in K48-ub to the increase in proteasome activity. However, it is hard to imagine that a mere few % increase in proteasome activity drastically reduces the accumulation of ubiquitinated proteins. There is no evidence showing that this increase in proteasome activity is involved in the clearance of ubiquitinated proteins. The y-axes of Figures 4D and 4G and 4Q should start at zero.

Response: As suggested by reviewer 1, the original figure 4 has been removed. These data are not included in the manuscript.

13. Fig 4N: The authors should explain why there was no difference between WT and ERα KO in female mice. The authors conclude that ERα promotes the activity of proteasome under mitochondrial proteotoxic stress, but there is no evidence. To conclude this, the authors should examine whether the increase in proteasome activity by Dox {plus minus} HS treatment is canceled in ERα KO mice. Also, the statement in the abstract "Importantly, activation of the estrogen receptor ...in critical to enhance proteasome activity and rescue misfolded protein accumulation in ALS spinal cords" is not at all supported by the data.

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14. Suppl Fig 7: It is interesting that female mice are more capable of activating mitochondrial stress response genes. However, the data show poor response to Dox in contrast to a strong response to HS. The authors should explain this point. Also, statistical analysis should compare between male and female. In the text, the authors should spell out NRF1, OMI1, and ATF.

Response: Since this part of discussion on the UPRmt has been removed, suppl. figure 7 has also been removed.

15. What is "LC" in Fig 3CG and 4CFPQ?

Response: LC stand for loading control, it was changed to actin.

16. The title is an overstatement. The authors did not show susceptibility to protein aggregations in "other tissues."

Response: as suggested, “other tissues” was changed to “intestine” in the title.

Referee #3:

The results of this manuscript reveal a sexual and tissue-specific difference in proteasome activity, which was already reported in the literature but only a small subset of tissues was tested. This study is more extensive which is of great interest to better understand how proteostasis is differentially regulated in different tissues and gender. The disease-related part is interesting but lacks statistical power to me, which associated to the experimental variability, sounds a bit less convincing. The importance of the ERalpha axis for the doxycycline regulation of proteasome activity also need to be strengthened. Proteostasis regulation in different genders and in pathophysiological conditions is of

great interest to the field, but to me, the weaknesses of this study need to be addressed before being considered for publication.

Specific comments:

1. The authors have measured proteasome activity in vitro with or without the proteasome inhibitor bortezomib only in Suppl. Fig. 1, while all experiments should have been conducted +/- proteasome inhibitors to be more accurate. In the same line, one of the big problems for the identification of ubiquitinated proteins in cellular lysates is the high activity of deubiquitinases (DUBs). The authors have analysed the load of K48-linked polyubiquitin chains without DUBs inhibitors (50mM Tris, 250mM NaCl, 5mM EDTA, 0.5% NP-40, 50mM NaF, 1mM DTT plus protease inhibitors) which could be one possible explanation for the observed variability among mice groups.

Response: We have repeated the experiment with and without proteasome inhibitors in the spinal cord. Since the intestine and the spinal cord are the focus of this study, we trust that having new data on both tissues with and without proteasome inhibitors will satisfy the reviewer. In addition, we have repeated the experiments in both intestine and spinal cord with and without DUB inhibitor. This new data is shown in new supplementary fig. 2. The pattern of Ub-K48 remains the same.

2. The authors concluded that the differences in activity are not due to the amount of proteasome by monitoring the levels of beta5 subunit, but the total level of proteasome subunits doesn't always correlate with proteasome levels as these subunits might belong to the pool of unassembled subunits, proteasome intermediates, free 20S proteasomes or singly- and doubly-capped proteasomes. Native gels should be able to confirm their statement, as the different forms of proteasomes can be identified.

Response: We agree entirely with the reviewer on this point and recognize that there are addiontal layers of comletxity in addition to subunit expression that would influence proeosome activity. Native gels were performed in both intestine and spinal cords to gain some insight on this point. Additionally, we added language the the text to recognize this complexity. Data is now shown in new figure 2F and G and the quantification shown in 2H and I.

2. The authors state: "Collectively these results suggest that sex specific differences in proteasome activity translate into a higher susceptibility to accumulate protein aggregates in males under heat shock stress (Fig. 3J)"; however, no real evidence indicate that this statement in true, as protein aggregates are mainly in the insoluble fractions and no significant differences are observed between insoluble fractions from male and female intestines while this tissue harbours the biggest gender difference in proteasome activity. This should be discussed by the authors.

Response: the sentence that summarizes the findings now shown in figure 4 was changed to: "*These results suggest that small sex-specific differences in proteasome activity in the spinal cord translate into a higher susceptibility to accumulate protein aggregates in males under heat shock stress.*"

3. The authors state: "Taking advantage of samples obtained from spinal cords of ERalpha knockout mice28, we confirmed that in absence of ERalpha spinal cord proteasome activity is decreased (Fig. 5N). This observation therefore confirms that the ERalpha promotes the activity of the proteasome under mitochondrial proteotoxic stress". As the authors have not tested proteasome activity in WT and ERalpha KO mice under mitochondrial stress, thus this statement needs to be modified.

Response: As suggested by reviewer 1, the data related to dox and ERalpha was removed and therefore this statement is no longer present.

4. The authors see a drastic effect of doxycycline on the load of K48-linked ubiquitin chains (Fig. 4B) while the activity of proteasome increases only by about 4% in female (Fig. 4D). It is clear that a small modification of proteasome activity can have important effects on the clearance of proteins but an alternative scenario is that doxycycline by inhibiting mitochondrial translation and possibly cytosolic translation (Mortison et al, Cell Chemi. Biol. 2018), decreases the synthesis of unwanted proteins and therefore their subsequent K48-specific ubiquitinylation. This could be discussed in the discussion part.

Response: We agree. While the data is no longer part of the current manuscript, we plan on expanding the analysis of Dox and proteasome in future work, and we will keep this important comment in mind. We thank the reviewer to point this out.

Minor comments:

1. The authors state: "Collectively, this analysis revealed that the decline in proteasome activity during aging is not uniform among tissues or sexes and that the overall decline is less pronounced in females than in males as reduction in proteasome activity is observed in 4 tissues in females but in 6 tissues in males (Fig. 2E, F)". This is not appropriate to me, as there are 3 tissues where proteasome activity is increased in old female, while 6 are increased in older males. Thus, the opposite conclusion could also be made. Moreover, the Fig. 2F shows that trypsin-like proteasome activity is decreasing in old males compared to younger males, while based on Suppl. Fig. 4B, it is the opposite. This, need to be corrected.

Response: The sentence: Collectively, this analysis revealed that the decline in proteasome activity during aging is not uniform among tissues or sexes and that the overall decline is less pronounced in females than in males as reduction in proteasome activity is observed in 4 tissues in females but in 6 tissues in males **was changed to**: Collectively, this analysis revealed that the decline in proteasome activity during aging is not uniform among tissues or sexes.

2. The authors say that HS in mice mimics high fever and this needs to be supported by references.

Response: This statement was made by inference, because 40C in humans corresponds to high fever and the normal body temperature in mice (measured by rectal probe) is approximately 36C, but a reference to fever conditions in mice could not be found in pubmed, so the sentence was removed.

3. It is not clear whether statistical analyses are missing in Suppl. Fig. 5B and 5D or whether the results are not significant.

Response: Statistics were added.

2nd Editorial Decision 6 December 2019

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, referee 1 and 3 acknowledge that the manuscript has been improved during the revision. However, I also note that several important concerns remain.

Referee 1 and 3 point out that several conclusions are not sufficiently supported by the data and respective overstatements need to be toned down. These relate to data showing that (1) ubiquitination differences reflect differences in proteasome activity, (2) beta 5 subunit levels reflect proteasome levels and (3) aggregates are caused by differences in proteasome activity.

Text and figures should be clarified according to the suggestions from referee 1.

In addition, referee 3 points out that the concentration of Bortezomib (2 mM) is likely to cause off-target effects (point 1). In case you indeed used 2 mM Bortezomib, the experiments need to be repeated with a lower concentration.

Moreover, evidence needs to be provided that the applied heat shock effectively changed the body temperature of the mice. This can be either a reference to a paper that established this method and provides clear evidence that the applied measure has an effect on mouse body temperature or by providing measurements of mouse body temperature during heat shock, which is preferred (referee 3, point 5).

Referee 2 remains concerned that caspase-like activity and the activity of the immunoproteasome have not been assessed. Upon further discussion, the other referees agreed with this point but considered it not essential. Referee 2 suggested however to probe the levels of beta1i, beta2i and/or beta5i in different tissues from male and female. In either case, this limitation should be clearly discussed in the manuscript.

To conclude, given the support from at least two referees, we would like to invite you to further revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

6) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

8) Other thing we need from the editorial side:

a) Your article will be published as 'Scientific report'. Please combine the Results and Discussion section and please note that the main text should not exceed 25,000 (+/- 2,000) characters, excluding references and materials and methods.

b) The title may not exceed 100 characters incl. spaces.

c) Please update the references to the numbered format of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors. You can download the respective EndNote file from our Guide to Authors
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

d) Please include a conflict of interest statement in the article.

e) Figure legends: for all quantifications, please define the meaning of bars and error bars in the respective legend. Also the number of experiments that the quantification is based on has to be specified in the legend (n, biological, technical replicate or number of mice). The statistical test used has to be given. If information applies to more panels in one figure, it can be specified under a "Data information" paragraph at the end of the legend.

f) Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (width x height). You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

9) As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

****

REFEREE REPORTS

Referee #1:

The revised version of Jenkins et al is quite improved. The narrative of the paper is more coherent and the data more convincing. As far as the figures, I thought that SFig 1b could be improved. This is intended as a three dimensional plot but it is difficult or to me impossible to perceive the third dimension. Perhaps a different graphic program would present the data better or alternatively you could have two graphs, one showing PC1 vs PC2 and another showing PC2 vs PC3. Concerning SFig 4, I wondered whether the results shown are gender-controlled. It would be worthwhile to track down the original data if it can be done. In Fig 2 some of the plots are potentially confusing. If I have it right, the line in 2E only represents data from males whereas data from both sexes are combined to derive line in panels 2J and 2K. I think the legend can be worded more clearly to highlight this different treatment of the data from panel to panel.

Minor comments on the text:

Page 3, last line: "cortical thymic epithelium" probably better than "thymus"

Page 4 line 7: delete comma

Page 4 line 21: "whole" activity, previously commented on by multiple reviewers, is still a problem here.

Page 5 line 16: delete comma

Page 6 line 21: The logic here is that the difference in overall ubiquitin conjugate levels could reflect differences in deubiquitinating (DUB) activity or proteasome activity, so that if DUB activity is not responsible then the problem must be the proteasome by default. But this is erroneous reasoning because it ignores other prominent possibilities such as differences in the activity of the ubiquitin conjugating factors or the availability of free ubiquitin for conjugation.

Page 8 line 1: Correlations may be either strong or weak. I don't think quantitative correlations should be labeled as direct or indirect.

Page 8 line 7: when a band shifts on a native gel it should be referred to as a change in electrophoretic mobility, not molecular weight. Native gels are very sensitive to parameters other than molecular weight such as shape and charge.

Page 12 line 4: need a period

Page 12 line 17: "know" should read "known"

Page 13 line 1: "stress-induced subunits" should read "stress-induced components"

Page 13 line 5: "supports" should read "support"

Referee #2:

In the revised/resubmitted manuscript, the authors removed the substantial part of interesting data (e.g., Fig 4, Supple Fig 7 in the original manuscript). The data regarding ERα KO mice was necessary to suggest a mechanism that could explain the difference in proteasome activity and capacity of proteostasis between males and females. Also, this reviewer does not feel that the authors respond to the concerns seriously. In sum, the manuscript has become too descriptive and superficial and includes little mechanistic insight, which excludes this manuscript from the candidate of EMBO Reports.

Other specific comments:
1. Response 1: If the authors think as " perhaps others have seen caspase-like activity in fresh tissues," the authors should use fresh tissues. Male mice may have higher caspase-like activity, which can change the conclusions of the manuscript.
2. Response 4: The authors did not perform the experiments requested in comment 4. Analysis of immunoproteasome is quite relevant to this manuscript because it significantly affects the peptidase activities of the proteasome.
3. Response 5: The authors did not perform the experiments requested in comment 5. This reviewer believes this point is essential to evaluate proteasome.

Referee #3:

The results of this manuscript reveal a sexual and tissue-specific difference in proteasome activity, which was already reported in the literature but only a small subset of tissues was tested. This study is more extensive which is of great interest to better understand how proteostasis is differentially regulated in different tissues and gender. Compared to the previous manuscript, this version is more

focused and then more efficient in delivering key information. Most overstatements have been removed and the data now better fit the conclusions. As proteostasis regulation in different genders and in pathophysiological conditions is of great interest to the field, I will support the publication of this manuscript if the authors take into consideration the following comments.

Specific comments:

1. The authors have measured proteasome activity in vitro with or without the proteasome inhibitor bortezomib in the intestine and in the spinal cord but the concentration of Bortezomib used (2 mM) is way too high. Important off-target effects are expected at this concentration as the IC50 of bortezomib in cells is around 7 nM.

2. This statement is wrong and has to be removed: 'To distinguish whether the level of ubiquitination reflects the activity of the proteasome or of de-ubiquitinated enzymes (DUB), we repeated the analysis in the presence of a DUB inhibitor. We found that DUB inhibition did not alter the pattern of Ub-K48 ubiquitinated proteins (Suppl. Fig. 2), indicating that the level of ubiquitination reflects the activity of the proteasome.' Indeed, proteasome activity rely on the DUB activity of Rpn11 and in a less extend to proteasome-associated DUBs such as USP14 and UCH37. The use of DUB inhibitor in lysate is to maintain the integrity of K48 Ubiquitin chains when samples are further analyzed by immunoblot.

3. This sentence is not accurate as measuring the level of one proteasome subunit, here β5, is not enough to monitor proteasome levels: 'Having established these unexpected differences, we next aimed at determining if they reflect differences in the amount of proteasome. Since the largest differences were observed for the chymotrypsin-like activity, we used an antibody against the β5 subunit to quantify the proteasome.'. The sentence can be changed (e.g., Having established these unexpected differences, we next aimed at determining if they reflect differences in the amount of proteasome subunits. Since the largest differences were observed for the chymotrypsin-like activity, we have monitored the levels of used an antibody against the β5 subunits to quantify the proteasome') or other proteasome subunits have to be monitored. Same for this sentence: 'Therefore, we concluded that the differences in activity are not due to the amount of (proteolytic) proteasome subunits.'

4. Loading native extracts from different tissues in the same native gel would be a great addition to highlight the tissue-specific difference in proteasome assembly which might correlate with the observed difference in proteasome activity.

5. I wonder if the authors have measured mice temperature after the 20 min at 40C? You may expect that internal temperature (blood temperature) doesn't raise too much as mammals have mechanism to maintain relatively constant internal temperature. The mice temperature has to be tested and specified at least in the M&M to be sure that tissues are subjected to heat shock.

6. The sentence: 'These results suggest that small sex-specific differences in proteasome activity in the spinal cord translate into a higher susceptibility to accumulate protein aggregates in males under heat shock stress (Fig. 4J).' is overstated to me as the difference in proteasome might not be the cause of the accumulation of aggregates. Doing correlation analysis and say that difference in proteasome activity correlate to protein aggregates would be more accurate.

7. Same as comment 3: 'Since we did not find correlations between proteasome activity and the amount of proteolytic proteasome subunits, our data suggest that...'.

8. Images of Fig 2A and 2F overlap with numbers.

2nd Revision - authors' response 24 December 2019

Point by point responses:

Referee #1:

The revised version of Jenkins et al is quite improved. The narrative of the paper is more coherent and the data more convincing. As far as the figures, I thought that SFig 1b could be improved. This is intended as a three dimensional plot but it is difficult or to me impossible to perceive the third dimension. Perhaps a different graphic program would present the data better or alternatively you could have two graphs, one showing PC1 vs PC2 and another showing PC2 vs PC3. Concerning SFig 4, I wondered whether the results shown are gender-controlled. It would be worthwhile to track down the original data if it can be done. In Fig 2 some of the plots are potentially confusing. If I have it right, the line in 2E only represents data from males whereas data from both sexes are combined to derive line in panels 2J and 2K. I think the legend can be worded more clearly to highlight this different treatment of the data from panel to panel.

Response:

- SFig. 1 has been reorganized into two graphs
- SFig. 4 the information on gender is not provided in this publicly available data set.
- The legend in Fig. 2E should have been the same as in panels J and K. We apologize for this omission, the legend was added.

Minor comments on the text:

Page 3, last line: "cortical thymic epithelium" probably better than "thymus"
Response: Thymus was changed for cortical thymic epithelium.

Page 4 line 7: delete comma
Response: Done

Page 4 line 21: "whole" activity, previously commented on by multiple reviewers, is still a problem here.
Response: whole was removed

Page 5 line 16: delete comma
Response: Done

Page 6 line 21: The logic here is that the difference in overall ubiquitin conjugate levels could reflect differences in deubiquitinating (DUB) activity or proteasome activity, so that if DUB activity is not responsible then the problem must be the proteasome by default. But this is erroneous reasoning because it ignores other prominent possibilities such as differences in the activity of the ubiquitin conjugating factors or the availability of free ubiquitin for conjugation.
Response: Reviewer 3 also had the same comment and requested the sentence to be removed. The text was changed to: Addition of a de-ubiquitinated enzymes (DUB) inhibitor did not altered the pattern of ubiquitination (Suppl. Fig. 2).

Page 8 line 1: Correlations may be either strong or weak. I don't think quantitative correlations should be labeled as direct or indirect.
Response: Direct was changed for strong

Page 8 line 7: when a band shifts on a native gel it should be referred to as a change in electrophoretic mobility, not molecular weight. Native gels are very sensitive to parameters other than molecular weight such as shape and charge.
Response: molecular weight was changed from electrophoretic mobility.

Page 12 line 4: need a period
Response: Done.

Page 12 line 17: "know" should read "known"
Response: Done.

Page 13 line 1: "stress-induced subunits" should read "stress-induced components"
Response: Done.

Page 13 line 5: "supports" should read "support"
Response: Done.

Referee #3:

The results of this manuscript reveal a sexual and tissue-specific difference in proteasome activity, which was already reported in the literature but only a small subset of tissues was tested. This study is more extensive which is of great interest to better understand how proteostasis is differentially

regulated in different tissues and gender. Compared to the previous manuscript, this version is more focused and then more efficient in delivering key information. Most overstatements have been removed and the data now better fit the conclusions. As proteostasis regulation in different genders and in pathophysiological conditions is of great interest to the field, I will support the publication of this manuscript if the authors take into consideration the following comments.

Specific comments:

1. *The authors have measured proteasome activity in vitro with or without the proteasome inhibitor bortezomib in the intestine and in the spinal cord but the concentration of Bortezomib used (2 mM) is way too high. Important off-target effects are expected at this concentration as the IC50 of bortezomib in cells is around 7 nM.*
Response: This experiment was performed and is now shown in SFig. 1A and B. In spinal cord , where the activity is low, 7nM Bortezomib completely inhibits the activity. In intestine however, where the activity is the highest of all tissues, 7nM only shows a trend toward inhibition but 70nM inhibits the activity.

2. This statement is wrong and has to be removed: 'To distinguish whether the level of ubiquitination reflects the activity of the proteasome or of de-ubiquitinated enzymes (DUB), we repeated the analysis in the presence of a DUB inhibitor. We found that DUB inhibition did not alter the pattern of Ub-K48 ubiquitinated proteins (Suppl. Fig. 2), indicating that the level of ubiquitination reflects the activity of the proteasome.' Indeed, proteasome activity rely on the DUB activity of Rpn11 and in a less extend to proteasome-associated DUBs such as USP14 and UCH37. The use of DUB inhibitor in lysate is to maintain the integrity of K48 Ubiquitin chains when samples are further analyzed by immunoblot.
Response: this sentence was removed. The text now says simply: Addition of a de-ubiquitinated enzymes (DUB) inhibitor did not alter the pattern of ubiquitination (Suppl. Fig. 2).

3. This sentence is not accurate as measuring the level of one proteasome subunit, here β5, is not enough to monitor proteasome levels: 'Having established these unexpected differences, we next aimed at determining if they reflect differences in the amount of proteasome. Since the largest differences were observed for the chymotrypsin-like activity, we used an antibody against the β5 subunit to quantify the proteasome.'. The sentence can be changed (e.g., Having established these unexpected differences, we next aimed at determining if they reflect differences in the amount of proteasome subunits. Since the largest differences were observed for the chymotrypsin-like activity, we have monitored the levels of used an antibody against the β5 subunits to quantify the proteasome') or other proteasome subunits have to be monitored. Same for this sentence: 'Therefore, we concluded that the differences in activity are not due to the amount of (proteolytic) proteasome subunits.'
Response: Both sentences were changed for those proposed by the reviewer.

4. *Loading native extracts from different tissues in the same native gel would be a great addition to highlight the tissue-specific difference in proteasome assembly which might correlate with the observed difference in proteasome activity.*
Response: These gels were performed and are now shown in new SFig. 4. The text now includes the following sentence: In addition, side by side analysis of spinal cord and intestine samples in females and males revealed that the levels of unassembled proteasome correlate with the low and high activity of the proteasome in these tissues respectively (SFig. 4). Further this analysis also highlighted additional differences in the assembled proteasomes between these two tissues (SFig. 4).

5. *I wonder if the authors have measured mice temperature after the 20 min at 40C? You may expect that internal temperature (blood temperature) doesn't raise too much as mammals have mechanism to maintain relatively constant internal temperature. The mice temperature has to be tested and specified at least in the M&M to be sure that tissues are subjected to heat shock.*
Response: Yes we had done the measurements but this information was not included in the original manuscript. We have added the information in the Material and Methods under Heat shock section. The temperatures prior and after heat shock are indicated.

6. *The sentence: 'These results suggest that small sex-specific differences in proteasome activity in the spinal cord translate into a higher susceptibility to accumulate protein aggregates in males under heat shock stress (Fig. 4J).' is overstated to me as the difference in proteasome might not be the cause of the accumulation of aggregates. Doing correlation analysis and say that difference in proteasome activity correlate to protein aggregates would be more accurate.*
Response: This sentence was changed to: These results suggest that small sex-specific differences in proteasome activity in the spinal cord correlates with accumulation of protein aggregates in males under heat shock stress (Fig. 4J).

7. *Same as comment 3: 'Since we did not find correlations between proteasome activity and the amount of proteolytic proteasome subunits, our data suggest that...'.*
Response: The sentence was changed to: Since we did not find correlations between proteasome activity and the amount of proteolytic proteasome subunits, our data suggest….

8. *Images of Fig 2A and 2F overlap with numbers.*
Response: There are 4 mice analyzed in 2A and 5 mice analyzed in 2F.

3rd Editorial Decision 23 January 2020

Thank you for your patience while we have editorially reviewed your revised manuscript. I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

1) Please provide an Author Contributions section in the manuscript

2) Please reformat the references to match the style of EMBO reports. The abbreviation 'et al' should be used if more than 10 authors and the first 10 authors should be listed. Also the callouts to the references need to be numbers in square brackets and not superscripted.
ou can download the respective EndNote file from our Guide to Authors
https://drive.google.com/file/d/0BxFM9n2lEE5oOHM4d2xEbmpxN2c/view

3) Appendix: Please add a title page with a table of content including page numbers.

4) Our data editors have inspected the figure legends for completeness and accuracy and have specified the required changes in your manuscript text file in our online submission system. Please review and implement the changes and upload again a file with track changes. I have also made some changes to the abstract.

5) Please apply the same rules to the figure legends in the Appendix. Please define error bars and central lines, e.g. mean +/-SD. Furthermore, please define the nature of the replicates, i.e. technical or biological and specify the statistical test used (if applicable). This applies to Figures S1A, C, D, E. S2B, D. S3B. S4B. S6. S7B, D. S8

6) Could you please also try to shorten the title to 100 characters incl. spaces?

7) Finally, I made some changes to the synopsis text. Could you please review the attached document and let me known whether you agree?

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

https://embor.msubmit.net/cgi-bin/main.plex?el=A5Ij7pT1A5PDm2J4A9ftd62GiDvocH3ZzVRRybLe3gY

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

3rd Revision - authors' response 24 January 2020

The authors performed all minor editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Doris Germain
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2019-48978

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

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http://www.ncbi.nlm.nih.gov/gap

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The samples sizes were not pre-specificed since we did not know if any differences were to be observed. However, samples size of 10 and 9 mice per group have allowed us to detect statistical significant differences between groups and are therefore appropriate.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We estimated that a sample size of 9-10 mice per groups would be sufficient to detect differences between groups.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The inclusion criteria were age and sex of mice and these criteria were pre-established.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Mice of specific age and sex were randomized to treatment groups.
For animal studies, include a statement about randomization even if no randomization was used.	Other than bering selected by age and sex, the mice were randomized to treatment groups.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not used.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes

Is there an estimate of variation within each group of data?	No, but variation was observed in selected groups and reported as such.
Is the variance similar between the groups that are being statistically compared?	No, some groups shpw much more variance than others.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	K48Ub (EMD-Millilore, cat. No. 05-1307), Actin (Santa Cruz Biotechnology, cat. No. sc-47778), β5 proteasome subunit (Enzo life sciences cat No. BML-PW8895-0025), Tubulin (Uoflowa, cat No. 12G10)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This study did not used cell lines

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Wild-type FVBN males and females, 3, 5, 10 and 15 months, B6SJL-TG_SOD1*G93A)1Gur/J and Non-transgenic (NTG) B6SJL males and females, and the transgenic SOD1-G93A in the latter background
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments were approved by the Mt. Sinai Institutional Animal Care and Use Committee (IACUC) and performed according to the principles of laboratory animal care outlined in NIH publication No. 86-23, revised 1985 edition
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	na
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	na
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	na
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	na
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	na
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	na
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	na

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	na
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	na
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	na
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	na

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	na